

Antibacterial activity of *Tabebuia impetiginosa* Martius ex DC (Taheebo) against *Helicobacter pylori*

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Abstract

The growth-inhibiting activity of *Tabebuia impetiginosa* Martius ex DC dried inner bark-derived constituents against *Helicobacter pylori* ATCC 43504 was examined using paper disc diffusion and minimum inhibitory concentration (MIC) bioassays. The activity of the isolated compounds was compared to that of the commercially available anti-*Helicobacter pylori* agents, amoxicillin, metronidazole, and tetracycline. The biologically active components of *Tabebuia impetiginosa* dried inner bark (taheebo) were characterized by spectroscopic analysis as 2-(hydroxymethyl)anthraquinone, anthraquinone-2-carboxylic acid, and 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol). With the paper disc diffusion assay 2-(hydroxymethyl)anthraquinone exhibited strong activity against *Helicobacter pylori* ATCC 43504 at 0.01 mg/disc. Anthraquinone-2-carboxylic acid, lapachol and metronidazole were less effective, exhibiting moderate anti-*Helicobacter pylori* activity at 0.1 mg/disc. Amoxicillin and tetracycline were the most potent compounds tested, displaying very strong activity at 0.005 mg/disc. 2-(Hydroxymethyl)anthraquinone exhibited moderate activity at this dose. Tetracycline still had strong activity at 0.001 mg/disc while amoxicillin had little activity at this dose. In the MIC bioassay, 2-(hydroxymethyl)anthraquinone (2 µg/mL), anthraquinone-2-carboxylic acid (8 µg/mL), and lapachol (4 µg/mL) were more active than metronidazole (32 µg/mL) but less effective than amoxicillin (0.063 µg/mL) and tetracycline (0.5 µg/mL). The anti-*Helicobacter pylori* activity of seven 1,4-naphthoquinone derivatives (structurally related to lapachol), 1,4-naphthoquinone, 5,8-dihydroxy-1,4-naphthoquinone (naphthazarin), 2-methyl-1,4-naphthoquinone (menadione), 2-hydroxy-1,4-naphthoquinone (lawsone), 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin), 5-hydroxy-1,4-naphthoquinone (juglone), and 2,3-dichloro-1,4-naphthoquinone (dichlone) was also evaluated using the paper disc assay. Menadione and plumbagin were the most potent compounds tested with the later still exhibiting very strong activity at 0.001 mg/disc. Menadione, juglone and tetracycline had strong activity at this low dose while the latter two compounds and amoxicillin had very strong activity at 0.005 mg/disc. Lawsone was unusual in that it had very strong activity at 0.1 and 0.05 mg/disc but weak activity at doses of 0.01 mg/disc and lower. Naphthazarin, lapachol and dichlone had similar activities while metronidazole had the lowest activity of all compounds tested. These results may be an indication of at least one of the pharmacological actions of taheebo. The *Tabebuia impetiginosa* dried inner bark-derived materials, particularly 2-(hydroxymethyl)anthraquinone, merit further study as potential *Helicobacter pylori* eradicating agents or lead compounds.

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Keywords: *Tabebuia impetiginosa*; Taheebo; *Helicobacter pylori*; 2-(Hydroxymethyl)anthraquinone; Anthraquinone-2-carboxylic acid; Lapachol; 1,4-Naphthoquinone derivatives

1. Introduction

In humans, *Helicobacter pylori* is a microaerophilic gram-negative bacterium that colonizes the stomachs of about half of the population (Dunn et al., 1997). *Helicobacter pylori* infection is recognized as an important casual agent in gastroduodenal disease such as chronic gastritis, peptic ulceration, gastric

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adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (MALToma) (Graham, 1997; Raus et al., 1987; Wotherspoon et al., 1993). The significance of these infections and the need for effective therapeutic agents have led to the development of several drug treatment regimens including a mixture of two antibiotics such as amoxicillin, clarithromycin, and/or metroidazole with bismuth or a proton pump inhibitor, which are still the most effective drugs (Dunn et al., 1997; Edwards, 1993). Although these treatments have been effective in attaining high cure rates, repeated use of these chemical drugs may lead to development of resistance and resistant strains (Becx et al., 1990; Matsumoto et al., 1997) and eradication failure rates range from 5 to 20%. Such treatments have had undesirable effects on nontarget organisms such as intestinal microorganisms (Ahn et al., 2000) and have sometimes caused serious side effects such as diarrhea, nausea, abnormal taste, dyspepsia, abnormal pain/discomfort, headache, and angiodema. Therefore, there is a strong demand for compositions having all of the beneficial properties of current therapy agents but with fewer side effects. Antimicrobial substances other than antibiotics would be very useful in the treatment of *Helicobacter pylori* infection if they were shown to be effective against both antibiotic-resistant and susceptible *Helicobacter pylori* strains.

Plants, particularly higher plants, may be an alternative source of materials for *Helicobacter pylori* eradication because they constitute a rich source of bioactive chemicals (Wink, 1993). In fact, a number of drugs and natural substances such as ecabet sodium, tea catechins, garlic extracts and honey have been shown to have antibacterial effects against *Helicobacter pylori* in vitro (Mabe et al., 1999; Ohta et al., 1999; Osato et al., 1999; Shibata et al., 1995). In the present study, we investigated the inhibitory effect of extracts from the inner bark of *Tabebuia* spp. (Bignoniaceae) against *Helicobacter pylori*. *Tabebuia* spp. are native to Central and South American tropical rain forests. The herbal product obtained from the bark of *tabebuia* trees is called taheebo or pau d'arco. The material is traditionally used for treating ulcers, syphilis, gastrointestinal problems, candidiasis, cancer, diabetes, prostatitis, constipation, and allergies. Many studies on the biological and pharmacological effects of *Tabebuia* spp. extracts have been detailed. The antineoplastic and antitumor-promoting effects of *Tabebuia avellanedae* Lor. ex Griseb. (de Santana et al., 1968; Udea et al., 1994), the cytotoxicity of *Tabebuia cassinoides* (Rao and Kingston, 1982), and the antitumor activity of *Tabebuia barbata* (Saizarbitoria et al., 1992) have been described.

To our knowledge, several pharmacological actions of taheebo have been reported to date. However, its anti-*Helicobacter pylori* property has not been elucidated. In this study, the active principles isolated from taheebo were characterized by spectroscopic analysis and their antibacterial activity against *Helicobacter pylori* was determined using paper disc diffusion and minimum inhibitory concentration (MIC) bioassays. Additionally, for the elucidation of structure–activity relationships, anti-*Helicobacter pylori* activities of commercially available 1,4-naphthoquinone derivatives are also presented. We hope that these studies will lead to the development of new and safer types of anti-*Helicobacter pylori* agents.

2. Materials and methods

2.1. Plant material

The dried inner bark of *Tabebuia impetiginosa* Mart. ex DC was purchased from Frontier Natural Products Co-op (Norway, IA).

2.2. Chemicals

Anthraquinone-2-carboxylic acid, dichlone, 2-(hydroxymethyl)anthraquinone, juglone, lapachol, lawsone, menadione, 1,4-naphthoquinone, naphthazarin, and plumbagin were purchased from Aldrich Chemical Co. (Milwaukee, WI). *N,N*-Dimethylformamide (DMF) was obtained from EM Science (Gibbstown, NJ). Clarithromycin was obtained from Abbott Laboratories Ltd. (Queensborough, UK). Amphotericin B, metronidazole, polymyxin B, trimethoprim, tetracycline, and vancomycin were purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

2.3. Bacterial strain and culture conditions

The test strain *Helicobacter pylori* ATCC 43504 used for the bioassay was obtained from the America Type Culture Collection (Rockville, MD). Stock cultures of the strain were routinely stored at -80°C on Brucella broth (Difco, Detroit, MI) containing 5% bovine calf serum (Hyclone, Longan, UT) and 20% glycol and when required were subcultured on Brucella agar. The plates were incubated at 37°C for 3–5 days in an atmosphere of 5% O_2 , 15% CO_2 , and 80% N_2 in an anaerobic chamber (Hirayama, Tokyo, Japan). On the following day, the organism was grown in Brucella broth (pH 7.4) with some supplements, which contained 5% bovine calf serum, $10\text{ }\mu\text{g/mL}$ of vancomycin, $5\text{ }\mu\text{g/mL}$ of polymyxin B, $5\text{ }\mu\text{g/mL}$ of trimethoprim, and $2\text{ }\mu\text{g/mL}$ of amphotericin B. To maintain a moist atmosphere, a moist paper towel was placed in the jar. All cultures were checked for contamination at the end of the growth cycle.

2.4. Isolation and identification

The dried inner bark of *Tabebuia impetiginosa* Mart. ex DC (3.0 kg) was extracted two times with methanol (25 L) at room temperature for 2 days and the extract was filtered. The resultant extract was combined and concentrated under reduced pressure at 40°C to yield about 12.13% (based on the weight of the dried inner bark). The methanol extract (50 g) was sequentially partitioned into hexane (9.9 g), chloroform (4.6 g), ethyl acetate (6.4 g), butanol (13.4 g), and water-soluble (15.7 g) fractions for bioassay. The organic solvent fractions were concentrated to dryness by rotary evaporation at 40°C , while the water fraction was freeze-dried.

The chloroform fraction (10 g) was chromatographed on a silica gel column (70–230 mesh, 500 g, $5.5\text{ cm} \times 70\text{ cm}$; Merck, Darmstadt, Germany), and successively eluted with a stepwise gradient of chloroform/methanol (100/0, 90/10, 80/20, 70/30, 60/40, 50/50 and 0/100 v/v). Column fractions were analyzed

by thin-layer chromatography (TLC; Silica gel G), and fractions with a similar TLC pattern were pooled. The bioactive fraction (1.5 g) was successively rechromatographed on a silica gel column, using a stepwise gradient of chloroform/methanol (95/5, 90/10, 80/20, 70/30, and 0/100 v/v). For further separation of the constituents, the active 95/5 fraction (600 mg) was fractionated by preparative high-performance liquid chromatography (HPLC; Spectra System P2000, Thermo Separation Products, San Jose, CA). The column was a 250 mm × 4.6 mm i.d. Cosmosil 5C₁₈-MS-II (Nacalai Tesque, Kyoto, Japan). A linear gradient of methanol–acetonitrile–0.1% H₃PO₃ (25:20:55 v/v; isocratic for 5 min) to methanol–acetonitrile–0.1% H₃PO₃ (25:45:20 v/v) in 20 min at a flow rate of 1.3 mL/min was used. The column effluent was detected at 254 nm. Finally, three active principles 1 (0.81 mg), 2 (0.75 mg), and 3 (1.25 mg) were isolated. The procedure was repeated three times to provide sufficient amounts of each constituent for structural determination. Structural determination of the active isolates was made by spectroscopic analysis. ¹H and ¹³C Nuclear magnetic resonance (NMR) spectra were obtained at 298 K with TMS as an internal standard on a Bruker model ARX400 spectrometer (Billerica, MA) at a frequency of 100.62 MHz for carbon and 400.13 MHz for proton. The spectrometer was equipped with a 5 mm ¹H/¹³C dual probe. The number of attached protons for ¹³C signals was determined from DEPT90 and DEPT135 assays. COSY and HMQC spectra were acquired using standard Bruker software. Fourier transform-infrared (FT-IR) spectra were obtained on a VECTOR 22 spectrometer (BRUKER, Frankfurt, Germany). The HPLC system (model HP1100) from Hewlett Packard (Agilent, Waldbronn, Germany) consisted of a binary pump (model G1312A) and an autosampler (model G1313A). The analytical HPLC column (XterraTMC18, average particle size 3.5 μm, 150 mm × 2.1 mm i.d.) was purchased from Waters (Milford, MA). The mobile phase was a mixture of acetonitrile/water (95:5, v/v) with a flow rate of 0.3 mL/min at room temperature. The mobile phase was filtered through a 0.45 μm Whatman nylon membrane filter (Maidstone, UK) and degassed under vacuum before use. Mass spectrometric detection was performed using an ion-trap mass spectrometer (Finnigan LCQ; San Jose, CA) equipped with an electrospray source. The ESI source was operated under the following conditions: heated capillary temperature 200 °C, the nitrogen sheath gas 70 psi, and the auxiliary gas at 10 units.

2.5. Microbiological assay

Helicobacter pylori ATCC 43504 was incubated microaerobically on Brucella agar that was supplemented with 5% bovine calf serum at 37 °C for 3 days in anaerobic jars (Hirayama, Tokyo, Japan). The colonies were suspended in 10 mL Brucella broth. The inoculum (0.1 mL) was prepared to contain 1 × 10^{7–8} CFU/mL by adjusting the turbidity of the suspension. An impregnated paper disc bioassay was used for the anti-*Helicobacter pylori* activity of the test materials. The samples were tested using different amounts. A sample in 0.1 mL of methanol was applied by a microsyringe to the paper discs (ADVANTEC, 8 mm diameter and 1 mm thickness, Toyo Roshi,

Japan). After drying in a fume hood, the discs were placed on the agar surface that was inoculated with *Helicobacter pylori*. All of the plates were incubated at 37 °C for 3 days under microaerophilic conditions in anaerobic jars. Diameters of the inhibition zones were recorded. The control discs received 0.1 mL of methanol. All of the inhibition tests were replicated at least three times.

The antibacterial activity was classified as follows: very strong response, zone diameter ≥30 mm; strong response, zone diameter 21–29 mm; moderate response, zone diameter 16–20 mm; weak response, zone diameter 11–15 mm; and little or no response, zone diameter ≤10 mm (Lee et al., 2004).

The MIC (minimum inhibitory concentration) was determined by the agar dilution method (Malekzadeh et al., 2001). The MIC was determined by adding various concentrations (0.6–128 μg/mL) of the isolated compound and the three antibiotics to solid media before inoculation with *Helicobacter pylori* suspension (100 μL). A final inoculum of 10⁶ CFU/mL was inoculated onto Brucella agar. Plates were then incubated at 37 °C for 3 days under the same conditions mentioned above. The MIC was defined as the lowest concentration at which no visible growth was observed. Tetracycline, metronidazole, amoxicillin served as standards for comparison in antibacterial activity tests.

3. Results

3.1. Identification

Fractions obtained from the methanol extract of taheebo were assayed against *Helicobacter pylori* using the impregnated paper disc method. Significant differences were observed in antibacterial activity against the tested fractions. The antibacterial activities of various fractions of taheebo (at concentrations of 5, 1, 0.5, 0.1, and 0.05 mg/disc) against *Helicobacter pylori* are shown in Table 1. The hexane and chloroform fraction exhibited very strong antimicrobial activity against *Helicobacter pylori* at the concentration of 0.5 and 0.1 mg/disc, respectively. The ethyl acetate fraction also showed very strong activity against *Helicobacter pylori* at a concentration of 5.0 mg/disc, whereas no inhibitory activities were produced by the butanol and water fractions. Due to its potent inhibitory activity against *Helicobac-*

Table 1

Antibacterial activity of each solvent fraction obtained from methanol extracts of the dried inner bark of *Tabebuia impetiginosa* toward *Helicobacter pylori* ATCC 43504 using the impregnated paper disc bioassay

Material	Inhibition zone (mm)				
	Dose (mg/disc)				
	5	1	0.5	0.1	0.05
Methanol extract	70	65	58	40	32
Hexane fraction	54	46	41	19	9
Chloroform fraction	64	59	52	32	24
Ethyl acetate fraction	31	15	9	– ^a	–
Butanol fraction	–	–	–	–	–
Water fraction	–	–	–	–	–

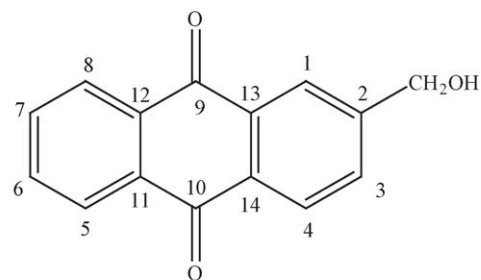
^a No inhibition observed.

Table 2

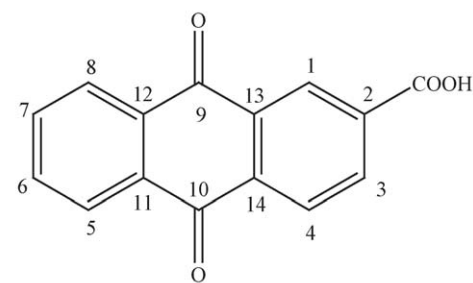
Antibacterial activity of chloroform subfractions derived from the dried inner bark of *Tabebuia impetiginosa* against *Helicobacter pylori* ATCC 43504 using the impregnated paper disc bioassay

Subfraction	Inhibition zone (mm)			
	Dose (mg/disc)			
	1	0.5	0.1	0.05
C1	8	8	8	8
C2	47	36	35	30
C3	37	34	25	19
C4	17	11	8	8
C5	17	11	8	8

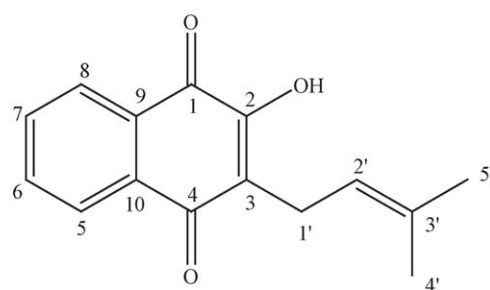
ter pylori, the chloroform fraction was selected for further study. The chloroform fraction was separated into five subfractions whose activities are given in Table 2. Very strong antibacterial activity was observed with subfraction C2 at 0.05 mg/disc. Silica gel column chromatography and preparative HPLC were used for further separation of the constituents in the C2 subfraction (1.02 g). The antibacterial activity-guided fractionation using *Helicobacter pylori* led to the isolation of three active principles that were identified as 2-(hydroxymethyl)anthraquinone (1), anthraquinone-2-carboxylic acid (2), and 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol) (3) (Fig. 1) by means of spectroscopic analysis and by direct comparison with authentic compounds. These three compounds were identified on the basis of the following evidence. **2-(Hydroxymethyl)anthraquinone:** ($C_{15}H_{10}O_3$, MW, 238.2); FT-IR: ν_{\max} (Nujol) 3527 (OH), 1675 (C=O), 1589 (aromatic rings) cm^{-1} , FAB-MS: m/z 239 $[M+H]^+$, LC-ESI-MS: m/z 239 $[M+H]^+$, EI-MS (rel. int. %): m/z 238 $[M]^+$ (80), 209 (100), 181 (24), 152 (50), 1H NMR (400 MHz, $CDCl_3$): 4.90 (2H, s, CH_2), 7.79 (2H, m, H-6,7), 7.81 (1H, m, H-3), 8.28 (1H, m, H-1), 8.30 (1H, m, H-4), 8.31 (2H, m, H-5,8), ^{13}C -NMR (100 MHz, $CDCl_3$): 183.15 (C, C-9), 182.92 (C, C-10), 147.64 (C, C-2), 134.17 (CH, C-7 or C-6), 134.10 (CH, C-7 or C-6), 133.72 (C, C-13), 133.62 (C, C-12 or C-11), 133.61 (C, C-11 or C-12), 132.80 (C, C-14), 131.99 (CH, C-3), 127.75 (CH, C-4), 127.29 (CH, C-8 or C-5), 127.27 (CH, C-5 or C-8), 124.95 (CH, C-1), 64.44 (CH_2). **Anthraquinone-2-carboxylic acid:** ($C_{15}H_8O_4$, MW, 252.2); FT-IR: ν_{\max} (Nujol) 1699 (C=O), 1678 (C=O) cm^{-1} , FAB-MS: m/z 253 $[M+H]^+$, LC-ESI-MS: m/z 253 $[M+H]^+$, EI-MS (rel. int. %): m/z 252 $[M]^+$ (100), 224 (36), 207 (33), 151 (35), 1H NMR (400 MHz, DMSO d_6): 7.95 (2H, m, H-6,7), 8.21 (2H, m, H-5,8), 8.28 (1H, d, $J=8.0$ Hz, H-4), 8.37 (1H, dd, $J=1.6, 8.0$ Hz, H-3), 8.64 (1H, d, $J=1.6$ Hz, H-1), ^{13}C NMR (100 MHz, DMSO d_6): 181.96 (C, C-9), 181.81 (C, C-10), 165.85 (C, COOH), 135.79 (C, C-14), 135.48 (C, C-2), 134.63 (CH, C-6 or C-7), 134.61 (CH, C-7 or C-6), 134.30 (CH, C-3), 133.11 (C, C-13), 132.96 (C, C-12), 132.96 (C, C-11), 127.28 (CH, C-1), 127.23 (CH, C-4), 126.76 (CH, C-8), 126.76 (CH, C-5). **2-Hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol)** ($C_{15}H_{14}O_3$, MW, 242.7); FT-IR: ν_{\max} (Nujol) 3350 (OH), 1660 (C=O), 1630 (aromatic rings) cm^{-1} , FAB-MS: m/z 243 $[M+H]^+$, LC-ESI-MS: m/z 243 $[M+H]^+$, EI-MS (rel. int. %): m/z 242 $[M]^+$ (37), 227 (100),



2-(hydroxymethyl)anthraquinone (1)



anthraquinone-2-carboxylic acid (2)



2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (3)

Fig. 1. Chemical structures of 2-(hydroxymethyl)anthraquinone (1), anthraquinone-2-carboxylic acid (2), and 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol) (3), antibacterial constituents from the dried inner bark of *Tabebuia impetiginosa*.

199 (35), 181 (32), 152 (37), 128 (35), 105 (35). 1H NMR (400 MHz, $CDCl_3$): 1.68 (3H, s, CH_3), 1.79 (3H, s, CH_3), 3.31 (2H, d, $J=7.2$ Hz, $-CH_2-$), 5.21 (1H, m, $-CH=$), 7.66 (1H, m, H-7), 7.74 (1H, m, H-6), 8.06 (1H, m, H-8), 8.12 (1H, m, H-5), ^{13}C NMR (100 MHz, $CDCl_3$): 184.54 (C, C-4), 181.73 (C, C-1), 152.70 (C, C-2), 134.84 (CH, C-6), 133.83 (C, C-3'), 132.99 (C, C-10), 132.85 (CH, C-7), 129.49 (C, C-9), 126.80 (CH, C-5), 126.06 (CH, C-8), 123.52 (C, C-3), 119.69 (CH, C-2'), 25.75 (CH_3 , C-5', *trans*), 22.65 (CH_2 , C-1'), 17.90 (CH_3 , C-4', *cis*).

3.2. Antibacterial activity

The anti-*Helicobacter pylori* activity of 2-(hydroxymethyl)anthraquinone, anthraquinone-2-carboxylic acid, and lapachol along with commercially available antibiotics such as amoxicillin, metronidazole, and tetracycline was evaluated

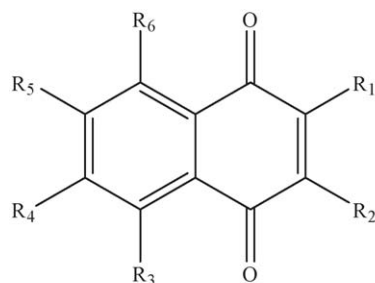
Table 3

Antibacterial activity of isolated constituents and antibiotics against *Helicobacter pylori* ATCC 43504 using the impregnated paper disc bioassay

Compound	Inhibition zone (mm)				
	Dose (mg/disc)				
	0.1	0.05	0.01	0.005	0.001
2-(Hydroxymethyl)anthraquinone	27	25	22	20	12
Anthraquinone-2-carboxylic acid	16	15	8	8	8
2-Hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol)	19	17	16	8	8
Amoxicillin	68	60	52	40	8
Metronidazole	16	8	8	8	8
Tetracycline	63	45	35	30	24

by comparing the inhibition zone diameters determined by the paper disc diffusion bioassay (Table 3). At 0.01 mg/disc, 2-(hydroxymethyl)anthraquinone exhibited strong activity against *Helicobacter pylori* ATCC 43504. Anthraquinone-2-carboxylic acid, lapachol and metronidazole were less effective, exhibiting moderate anti-*Helicobacter pylori* activity at 0.1 mg/disc. Amoxicillin and tetracycline were the most potent compounds tested, displaying very strong activity at 0.005 mg/disc. 2-(Hydroxymethyl)anthraquinone exhibited moderate activity at this dose. Tetracycline still had strong activity at 0.001 mg/disc while amoxicillin had little activity at this dose.

To assess structure-activity relationships, the anti-*Helicobacter pylori* activity of seven 1,4-naphthoquinone derivatives (Fig. 2) was compared to that of lapachol and three commercial antibiotics (Table 4). Menadione and plumbagin were the most potent compounds tested with the later still exhibiting very strong activity at 0.001 mg/disc. Menadione, juglone and tetracycline had strong activity at this low dose while the latter two compounds and amoxicillin had very strong activity at 0.005 mg/disc. Lawsone was unusual in that it had very strong activity at 0.1 and 0.05 mg/disc but weak activity at doses of 0.01 mg/disc and lower. Naphthazalin, lapachol and dichlone had similar activities while metronidazole had the lowest activity of all compounds tested.



R₁=R₂=R₃=R₄=R₅=R₆=H (1,4-naphthoquinone)
 R₁=R₂=R₄=R₅=H; R₃=R₆=OH (naphthazarin)
 R₂=R₃=R₄=R₅=R₆=H; R₁=CH₃ (menadione)
 R₂=R₃=R₄=R₅=R₆=H; R₁=OH (lawsone)
 R₂=R₄=R₅=R₆=H; R₁=CH₃; R₃=OH (plumbagin)
 R₁=OH; R₂=CH₂CH=C(CH₃)₂ (lapachol)
 R₁=R₂=R₄=R₅=R₆=H; R₃=OH (juglone)
 R₃=R₄=R₅=R₆=H; R₁=R₂=Cl (dichlone)

Fig. 2. Chemical structures of 1,4-naphthoquinone derivatives.

Table 4

Antibacterial activity of 1,4-naphthoquinone derivatives and antibiotics against *Helicobacter pylori* ATCC 43504 using the impregnated paper disc bioassay

Compound	Inhibition zone (mm)				
	Dose (mg/disc)				
	0.1	0.05	0.01	0.005	0.001
1,4-Naphthoquinone	52	47	31	28	16
Naphthazalin	29	29	23	22	13
Menadione	86	73	49	29	21
Lawsone	43	34	12	8	8
Plumbagin	72	63	61	50	47
Lapachol	27	25	22	20	12
Juglone	55	52	40	37	22
Dichlone	24	23	18	15	12
Amoxicillin	68	60	52	40	8
Metronidazole	16	8	8	8	8
Tetracycline	63	45	35	30	24

Table 5

Minimum inhibitory concentrations (MIC) of compounds isolated from taheebo and antibiotics against *Helicobacter pylori* ATCC 43504

Compound	MIC (μg/mL)
2-(Hydroxymethyl)anthraquinone	2
Anthraquinone-2-carboxylic acid	8
Lapachol	4
Plumbagin	4
Amoxicillin	0.063
Metronidazole	32
Tetracycline	0.5

The anti-*Helicobacter pylori* effect of the test compounds in the MIC assay is shown in Table 5. The MIC of 2-(hydroxymethyl)anthraquinone (1), anthraquinone-2-carboxylic acid (2), lapachol (3) were 2, 8, and 4 μg/mL, respectively. The 1,4-naphthoquinone analog, plumbagin, exhibited growth inhibition of *Helicobacter pylori* at 4 μg/mL. These compounds had stronger activity than metronidazole which had an MIC value of 32 μg/mL. Amoxicillin and tetracycline displayed the most potent activity, completely inhibiting *Helicobacter pylori* growth at 0.063 and 0.5 μg/mL, respectively.

4. Discussion

Helicobacter pylori has been implicated as being responsible for gastritis, duodenal ulcers, and possibly neoplasia.

Antibacterial treatment of *Helicobacter pylori* is difficult because of the habitat occupied by the organism below the layer of mucus adherent to gastric mucosa. The pH of gastric juice and sites within the mucosa may be an important factor that potentially affects drug activity. It is known that single agents are generally ineffective or poorly effective in eradicating *Helicobacter pylori*. In addition, the problem of resistance is very important since it readily occurs with monotherapy, which therefore should never be used for *Helicobacter*. The combination of two or more antibacterial agents is therefore recommended (Harris and Misiewicz, 1996). Access of antibacterial agents to this site is limited from the lumen of the stomach and also from the gastric blood supply. The ideal therapy for *Helicobacter pylori* eradication should be simple, safe, and free from side effects. It has been well recognized that plant extracts and phytochemicals could be developed into products suitable for *Helicobacter pylori* eradication because many of them are selective, often biodegrade to nontoxic products, and may be applied to humans in the same way as other conventional chemical drugs (Cowan, 1999). Many plant extracts and phytochemicals possess antibacterial activity against *Helicobacter pylori* (Tabak et al., 1996; Luigina et al., 1996; Marone et al., 2001). Additionally, some plant-derived materials have been reported to be active against *Helicobacter pylori* include capsaicin from *Capsicum annuum* (Nicola et al., 1997), anacardic acids (MIC, 200–800 $\mu\text{g/mL}$) derived from *Anacardium occidentale* apple (Kubo et al., 1999), cabreuvin (MIC, 30 $\mu\text{g/mL}$) from *Myroxylon peruiferum* (Ohsaki et al., 1999), six quinolone alkaloids (MIC, 10–20 $\mu\text{g/mL}$) such as evocarpine from the fruits of *Evodia rutaecarpa* (Rho et al., 1999), panaxytriol (MIC, 50 $\mu\text{g/mL}$) from *Panax ginseng* (Bae et al., 2001), and catechins from *Thea sinensis* (IC₅₀, 13 $\mu\text{g/mL}$) (Matsubara et al., 2003). The three constituents from taheebo, with MIC values ranging from 2 to 8 $\mu\text{g/mL}$, have activities that compare favorably to previously reported constituents.

Tabebuia spp. (Bignoniaceae) are native to tropical rain forests throughout Central and South America. Major constituents in bark extracts of *Tabebuia* spp. have been reported several times. They include furanonaphthoquinones (Diaz and Medina, 1996), quinones (Sharma et al., 1988), and naphthoquinones (Manners and Jurd, 1976). Naphthoquinones are widespread in nature and play important physiological roles in animals and plants. Secondary metabolites bearing in their structure the 1,4- and 1,2-naphthoquinone moieties have been isolated from plants and exhibit interesting biological activities. Lapachol and β -lapachone, found in species of *Tabebuia*, have relevant effects against *Candida albicans*, *Candida tropicalis*, and *Cryptococcus neoformans*, and were more active than the reference standard, ketoconazole. These results conformed that β -lapachone has more efficient antimicrobial activity than lapachol against the test fungi (Guiraud et al., 1994). It seems that lapachol acts by uncoupling oxidative phosphorylation, as do a few naphthoquinones which are also inhibitors. A large number of naphthoquinones are also inhibitors of electron transport (Howland, 1963). An analog of lapachol, “furanonaphthoquinone (FNQ)”, isolated from the tree bark of *Tabebuia impetiginosa* was found to possess a markedly low MIC value against *Helicobacter pylori*. MICs were determined for five strains of *Helicobac-*

ter pylori from different human specimens. FNQ inhibited the growth of those strains with an MIC of 0.1 $\mu\text{g/mL}$ (Nagata et al., 1998). No effect of culture medium pH values between 5.5 and 7.2 was found on the FNQ MIC for *Helicobacter pylori*. However, the MICs of some antibiotics are known to decrease 10–100-fold in acidic culture medium (pH 5.5) (Goodwin and McNulty, 1992). Since addition of FNQ to several antibiotics decreased their MICs against methicillin-resistant *Staphylococcus aureus* (MRSA), similar experiments were carried out with *Helicobacter pylori*. MICs of ampicillin, cefaclor, levofloxacin, minocycline and vancomycin were reduced 2–8-fold in the presence of FNQ (Nagata et al., 1998). The effects of anthraquinone compounds on *Escherichia coli* K12, *Pseudomonas aeruginosa* PAO1 and some strains of *Staphylococcus aureus* have been investigated. Among them, aloe-emodin, rhein, and emodin showed noticeable antibacterial effects on four strains of MRSA with MICs of 2–64 $\mu\text{g/mL}$ (Hatano et al., 1999). 2-(Hydroxymethyl)anthraquinone and anthraquinone-2-carboxylic acid had MIC values of 2 and 8 $\mu\text{g/mL}$, respectively, against *Helicobacter pylori*.

A novel benz[a]anthraquinone, YM-181741, isolated from the culture broth of actinomycete strain Q57219, showed selective anti-*Helicobacter pylori* activity with a MIC value of 0.2 $\mu\text{g/mL}$ (Taniguchi et al., 2002). Arylamine *N*-acetyltransferase (NAT) activities with *p*-aminobenzoic acid (PABA) and 2-aminofluorene (AF) were determined in *Helicobacter pylori* collected from peptic ulcer patients. The results indicated that there was decreased NAT activity associated with increased anthraquinone series compounds such as rhein, aloe-emodin, and emodin in *Helicobacter pylori* cytosols. Studies demonstrated that anthraquinone series compounds such as rhein, aloe-emodin, and emodin elicited dose-dependent bacteriostatic activity in the growth inhibition of *Helicobacter pylori* (Chung et al., 1998; Wang et al., 1998; Chung et al., 1997).

In the present study, methanol extract from the dried inner bark of *Tabebuia impetiginosa* exhibited potent antibacterial activity against *Helicobacter pylori*. The biologically active constituents were identified as 2-(hydroxymethyl)anthraquinone, anthraquinone-2-carboxylic acid, and lapachol. The anti-*Helicobacter pylori* activity of the isolated compounds was more effective than that of metronidazole but less effective than that of amoxicillin and tetracycline. The structure of the 1,4-naphthoquinone analogs had a dramatic effect of their anti-*Helicobacter pylori* activity. A methyl group in the C-2 position seemed to be an important structural feature as plumbagin and menadione had stronger activity than their counterparts, juglone and 1,4-naphthoquinone (which lack methyl groups in the C-2 position). Addition of a hydroxy group to the C-2 position of 1,4-naphthoquinone (to form lawsone) resulted in a loss of activity, particularly noteworthy at doses below 0.05 mg/disc. Juglone (which has a hydroxy group in the C-5 position) had slightly higher activity than 1,4-naphthoquinone. Addition of a hydroxy group to the C-8 position of juglone (to form naphthazarin) strongly reduced the activity. The addition of a 3-methyl-2-butenyl side chain at C-3 to lawsone (to form lapachol) reduced the activity at higher doses (0.1 and 0.05 mg/disc) but increased the activity at doses of 0.01 mg/disc and below.

For practical use of the *Tabebuia impetiginosa* dried inner bark-derived materials as novel antibacterial agents to proceed, further research is necessary on safety issues of the plant-derived materials for human health, their antibacterial mode of action, and effective formulations for improving the antibacterial potency and stability in human gastrointestinal tract.

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